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The stability of lipidic analogues of GnRH in plasma and kidney preparations: the stereoselective release of the parent peptide

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Abstract—The conjugation of a lipoamino acid to the N-terminus of Gonadotropin releasing hormone (GnRH) produces a lipophilic peptide from which the parent GnRH peptide is released into solution on treatment with plasma and kidney enzyme preparation. Our findings show that one stereoisomer of the Laa is cleaved very rapidly, providing a bolus dose of the peptide while the opposite stereoisomer is cleaved much more slowly, providing prolonged elevation of peptide concentration. The Laa-Glu linkage appears to act as a two phase prodrug system.

Numerous peptides, from simple dipeptides to complex proteins, have been and continue to be identified as potential pharmaceutical agents for a variety of diseases. However, for the majority of these compounds, their progression into the clinic is thwarted by the persistent hurdles of poor oral absorption and rapid enzymatic breakdown. Increasing the lipophilicity of a hydrophilic compound has been established as an important factor in improving absorption via passive transport across intestinal mucosal membranes.[1] There are numerous reports of peptides that have been coupled to various lipid units, which has resulted in improved intestinal delivery characteristics.[2,3] However, any process which alters the native structure of the peptide can result in a significant drop in potency compared to the parent peptide.[4] The ideal solution to this is a prodrug strategy where the lipidic groups are conjugated to the peptide in order to aid their absorption and then removed in vivo to release the active peptide.

Gonadotropin releasing-hormone (GnRH, aka luteinizing hormone releasing hormone LHRH) is an endogenous decapeptide (Fig. 1) produced in the hypothalamus and stimulating the release of both luteinizing hormone and follicle-stimulating hormone from the anterior pituitary. GnRH and several of its analogues are used in the treatment of conditions such as endometriosis and hormone dependent cancers. Unfortunately the oral bioavailability of the peptide is very poor[5] and, once in the circulation, the peptide is very susceptible to enzymatic degradation.[6] We have reported previously that conjugating 2-amino-D,L-dodecanoic acid (Laa) to the N-terminus of the GluGnRH analogue (Fig. 1) can increase the half life of the peptide in an homogenate of Caco-2 cells from 5 min for the parent peptide to 45 min when conjugated to one Laa and 360 min when conjugated to two Laa residues.[7] These Laa-GluGnRH conjugates were absorbed across the epithelial barrier of the GI tract after oral administration to rats while the parent peptide was degraded rapidly in the gut.[8]

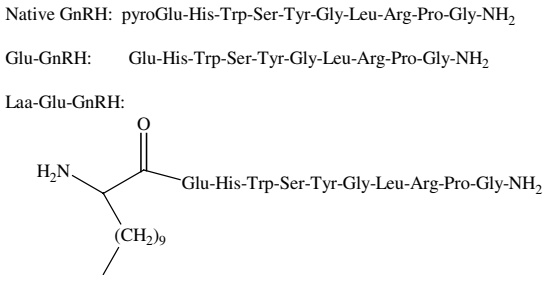

Figure 1. The sequence of GnRH, GluGnRH and Laa-GluGnRH.

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In our previous studies on the Laa-GluGnRH analogue it was apparent that treatment of this conjugate with an homogenate of Caco-2 cells caused GluGnRH to be released into solution. The diastereomeric compounds arising from the use of the D- or L-lipoamino acid clearly exhibited different susceptibility to enzymatic degradation with one epimer, presumably the L-isomer, being degraded much more rapidly than the D-epimer. There was also some evidence that the released GluGnRH exhibited increased stability suggesting that the presence of the lipoamino acid in solution could inhibit degradation.[7] This phenomenon represents a potentially useful prodrug strategy for peptide delivery and thus we proceeded to study the stability of Laa-GluGnRH in other biological media including plasma and kidney derived preparations.

The peptides were assembled on *p*-MBHA resin using HBTU/DIEA activation and the in situ neutralization protocol for Boc chemistry.[9] 2-(*tert*-Butoxycarbonylamino)-D,L-dodecanoic acid was synthesized via published methods from diethyl acetamido malonate and 1-bromodecane.[10] The racemic Boc-Laa was utilized in the peptide synthesis and upon cleavage of the peptides from the resin via standard HF protocols, the peptides were purified by RP-HPLC on a Vydac® C18 22×250 mm column using a linear solvent gradient from 100% solvent A to 80% solvent B (solvent A 0.1% TFA in water, solvent B 0.1% TFA, 90% acetonitrile in water) for 35 min at a flow rate 5 mL/min. Initially a racemic mixture of the two diastereomers was isolated but further HPLC succeeded in isolating the two diastereomers, termed E1 and E2. The identity and purity of the peptides was characterized by analytical HPLC using an Agilent-Zorbax® C18 4.6×150 mm column with a flow rate of 2 mL/min and a linear gradient from 100% solvent A to 80% solvent B over 9 min, and by LC–MS using an HPLC system (Shimadzu LC-10AT system) with an Agilent-Zorbax C18 2.1×50 mm column with a mobile phase consisting of a linear gradient of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in 90% acetonitrile/water) at 0.3 mL/min coupled to a triple quadrupole mass spectrometer (PE Sciex API 3000) operating in positive ion electrospray mode. The desired peptides were identified by the appearance of characteristic ESMS signals: $[M+H]^+$ ion (Laa-GluGnRH = 1398, GluGnRH = 1201) and the more abundant $[M+2H]^{2+}$ ion (Laa-GluGnRH = 699.5, GluGnRH = 601).

Laa-GluGnRH peptides (5 μg per time point) were incubated with platelet-poor rabbit plasma (6.4 or 1.25 μL per time point) or rabbit kidney membranes[11] (2.5 μg protein per time point) diluted in Tris-buffered saline (25 mM Tris–HCl, 125 mM NaCl, pH 7.4; 50 μL per time point). Samples taken at regular time intervals were stopped by addition of 4 volume methanol/1% trifluoroacetic acid (TFA), and dried on a centrifugal vacuum evaporator (Speed-Vac, Savant, Farmingdale, NY, USA) before HPLC analysis using a Agilent 1100 series LC equipped with a diode array detector and on-line electrospray mass spectrometric (ESMS) detector (Agilent Technologies, Palo Alto, CA, USA). Samples were loaded onto a Zorbax Eclipse C18 column (at 50 °C) in 1.8% acetonitrile/0.1% TFA/0.02% acetic acid at 0.15 mL/min, and eluted with a 30 min linear gradient to 60% acetonitrile/0.1% TFA. Peptide fragments were identified following on-line mass spectral analysis using Agilent ChemStation deconvolution software.

When a racemic mixture of D,L-Laa-GluGnRH was treated with plasma (1.25 μL plasma vs 5 μg peptide) the earlier eluting diastereomer, E1, was rapidly degraded to a single major peak exhibiting ESMS peaks corresponding to GluGnRH (1199.54) and GnRH 2–10 (1070.44). E1 was 50% degraded after only 1 h incubation while the alternate diastereomer, E2, was degraded to GluGnRH much more slowly, exhibiting a half-life of approximately 4 h. Figure 2 presents the HPLC trace of the mixture of diastereomers at time zero followed by the trace of the mixture after 30 min and then after 1 h of incubation. The traces clearly show the destruction of one of the isomers in preference to the other. The liberation of the parent Glu-GnRH peptide from the lipophilic conjugate indicates that the Laa-

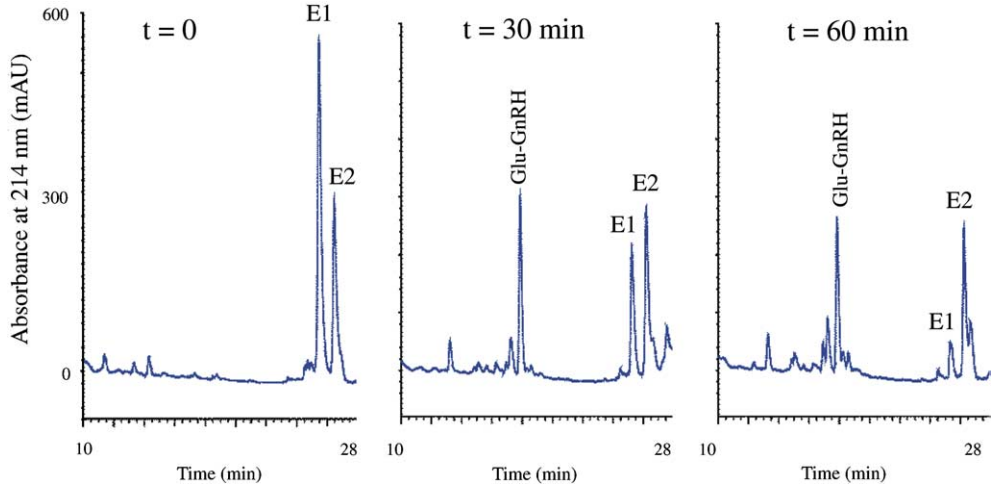

Figure 2. HPLC traces showing the rapid degradation of E1 and consequent release of GluGnRH in plasma.

Glu bond acts as a prodrug linkage and is cleaved by aminopeptidases in the plasma. The released Glu-GnRH is subsequently degraded by aminopeptidases producing the N-truncated peptide GnRH 2–10 and eventually the fragments 5–10, 6–10 and 7–10. The significance of this result is the large discrepancy in the rate of degradation of the two diastereomers. E1 is presumed to be L-Laa-GluGnRH but this is yet to be confirmed. The rapid degradation of E1 provides a bolus dose of the parent peptide into the system while E2, presumably D-Laa-GluGnRH, degradation provides a 'slow-release' of the parent peptide.

The question arises as to whether the delayed degradation of E2 is a consequence of simple competition with E1 or if it is a characteristic of the compound itself. To address this issue, the two diastereomers of Laa-Glu-GnRH were isolated by HPLC and treated individually with plasma (6.4 μL plasma vs 5 μg peptide). The results reflect those for the racemic mixture with E1 being degraded quickly to Glu-GnRH and GnRH 2–10 and exhibiting a half life of approximately 15 min. E2 exhibited significantly greater stability with a half life in excess of 2 h, the length of the experiment. Figure 3 clearly demonstrates the degradation profiles of the isolated compounds. It is clear, therefore that the stability of E2 is a feature of the compound, postulated to be due to the presence of the D-Laa, rather than due to its poor competition in the presence of E1 for the aminopeptidase enzymes.

It was observed in the original Caco-2 cell homogenate experiments that the GluGnRH liberated from the prodrug Laa-GluGnRH appeared to exhibit increased stability as the concentrations of this peptide remained high in solution even after 4 h while GluGnRH normally exhibited a half life in the cell homogenate of

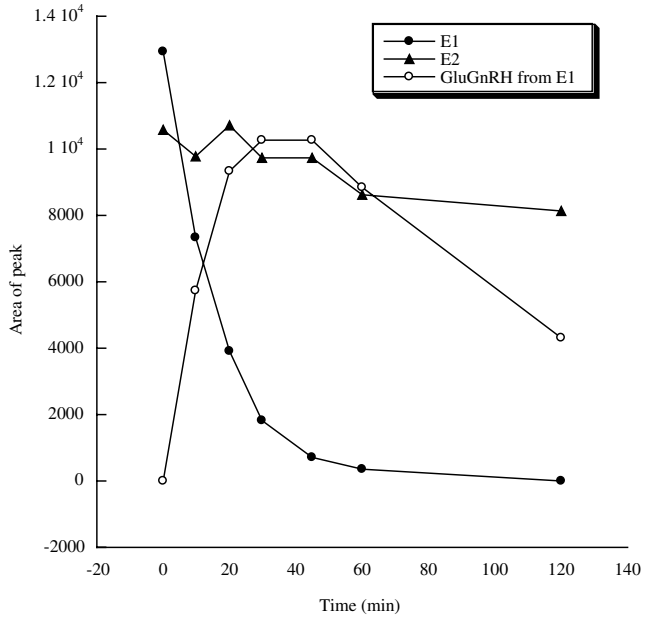

Figure 3. The degradation profiles of the isolated diastereomers in plasma overlaid with the concentration versus time curve for released GluGnRH.

~5 min.[7] It was postulated then that the presence of either 2-aminododecanoic acid, the released Laa or Laa-GluGnRH conjugate itself has an inhibitory effect on the aminopeptidases responsible for the destruction of GluGnRH. In order to test this hypothesis, both Glu-GnRH and E1 were treated with plasma in the presence and absence of 2-aminododecanoic acid (85 μM). There was no difference in degradation rate of either peptide in the presence of the Laa nor was there a difference in the rate of degradation of GluGnRH liberated from isolated E1. It appears that the prolonged elevation of Glu-GnRH concentration in the initial experiments is actually due to the slow release of GluGnRH from the E2 diastereomer, which provides a continuous source of GluGnRH over several hours.

The stability of the prodrug Laa-GluGnRH in a kidney membrane preparation was also analyzed in this study. When treated with 2.5 μg of protein from the kidney membrane preparation, D,L-Laa-GluGnRH exhibited a similar degradation profile as it did in plasma. E1 was degraded rapidly while E2 showed greater stability but was still degraded slowly. The peptides were degraded predominately to GluGnRH and GnRH 2–10 but other aminopeptidase cleavage fragments (GnRH 5–10, 6–10 and 7–10) were also present. The kidney membrane preparation was more efficient than plasma at degrading the peptide, releasing the parent Glu-GnRH but then efficiently destroying this peptide.

Our previous studies have shown that the presence of a lipoamino acid on the terminus of a peptide can increase the absorption of the peptide across plasma membranes[12] and that Laa-GluGnRH is absorbed into the circulation after oral administration to rats.[8] The present study clearly demonstrates that this orally absorbed compound is an effective prodrug for the parent GnRH peptide. The two diastereomers resulting from the use of both D- and L-Laa constitute a novel release system allowing for rapid bolus dosing from the L-epimer followed by slow release from the D-epimer. Although the native GnRH peptide contains pyroglutamic acid at the N-terminus, it is envisaged that GluGnRH will be converted in vivo to the active peptide by known cyclase enzymes.[13] The final test of the efficacy of this GnRH prodrug is the in vivo biological activity. The study of the effect of the compound dosed orally to male rats over 21 days is currently underway. A stereoselective synthesis of 2-aminododecanoic acid is also being developed in order to positively confirm the identity of E1 and E2 as the L- and D-Laa isomers respectively.

The stereoselective cleavage of the Laa-Glu bond by aminopeptidases is a phenomenon that may be of great importance to the development of prodrugs of other peptide based pharmaceutics. The process represents a widely applicable, two phase prodrug delivery system for peptides.

Acknowledgements

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References and notes

1. Bundgaard, H.; Hansen, B. B. *Pharm. Int.* **1981**(June), 136–140.
2. Blanchfield, J. T.; Toth, I. *Curr. Med. Chem.* **2004**, *11*, 2375–2382.
3. Wong, A.; Toth, I. *Curr. Med. Chem.* **2001**, *8*, 1123–1136.
4. Hashimoto, M.; Takada, K.; Kiso, Y.; Muranishi, S. *Pharm. Res.* **1989**, *6*, 171–176.
5. Sandow, J.; Petri, W. Intranasal Administration of Peptides, Biological Activity and Therapeutic Efficacy, In *Transnasal Systemic Medications*; Elsevier: Amsterdam, 1985; pp 183–199.
6. Handelsman, D. J.; Swerdloff, R. S. *Endocrinol. Rev.* **1986**, *7*, 95–105.
7. Toth, I.; Flinn, N.; Hillery, A.; Gibbons, W. A.; Artursson, P. *Int. J. Pharm.* **1994**, *105*, 241–247.
8. Flinn, N.; Coppard, S.; Toth, I. *Int. J. Pharm.* **1996**, *1996*, 33–39.
9. Schnolzer, M.; Alewood, P. F.; Jones, A.; Alewood, D.; Kent, S. B. H. *Int. J. Pept. Protein Res.* **1992**, *40*, 180–193.
10. Blanchfield, J.; Dutton, J. L.; Hogg, R.; Craik, D. J.; Adams, D. J.; Lewis, R.; Alewood, P. F.; Toth, I. *Lett. Pept. Sci.* **2002**, *8*, 235–239.
11. Lew, R. A.; Tomoda, F.; Evans, R. G.; Lakat, L.; Boublik, J. H.; Pipolo, L. A.; Smith, A. I. *Br. J. Pharm.* **1996**, *118*, 1269–1277.
12. Blanchfield, J. T.; Dutton, J. L.; Hogg, R. C.; Gallagher, O. P.; Craik, D. J.; Adams, D. J.; Lewis, R. J.; Alewood, P. F.; Toth, I. *J. Med. Chem.* **2003**, *46*, 1266–1272.
13. Fischer, W.; Spiess, J. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 3628–3632.